REMARKS

Claims 1-9, 11-17, 36 and 59 presently appear in this case. No claims have been allowed. The official action of July 5, 2005, has now been carefully studied. Reconsideration and allowance are hereby respectfully urged.

Briefly, the present invention relates to a method of altering gene expression in a population of human embryonic stem cells by introducing into the population of human embryonic stem cells a polynucleotide that is operably linked to a promoter and contains a gene expression altering sequence. It is possible to obtain a transfection efficiency greater than that obtainable by means of electroporation by use of a transfection reagent that is a cationic non-lipid polymer reagent, a non-liposomal reagent or a cationic lipid agent. The invention further relates to a substantially pure, stably transfected population of pluripotent human embryonic stem cells that have been modified to contain a gene expression altering sequence of DNA.

Claim 36 has been rejected under 35 U.S.C. §102(a) or 35 U.S.C. §102(e) as being anticipated by Smith because it teaches methods of transfection of any mammalian embryonic stem cell that include human ES cells. The examiner states that the claimed transfected cells do not depend on their method of production and, accordingly, the examiner maintains

that Smith anticipates the claimed invention. This rejection is respectfully traversed.

It is apparently the examiner's position that, if even one transformed human embryonic stem cell could be made using the process of Smith, it would anticipate claim 36. However, claim 36 is drawn to a "substantially pure stably transfected population of pluripotent human embryonic stem cells." Thus, the cell line must be substantially pure and stably transformed. The requirement that they be stably transformed means that transient transfection is insufficient and only transfection that has been shown to be stably maintained is claimed.

Those of ordinary skill in the art are aware that there is a concept of "dilution" when trying to maintain human embryonic stem cell lines. Human embryonic stem cell lines must be cultured on a culture medium with murine feeder cells. However, there is some kind of feedback mechanism between the human stem cells and the feeder cells. If the human stem cells are too diluted on the culture plate, they will not survive. In this regard, attached hereto is Amit et al, "Clonally Derived Human Embryonic Stem Cell Lines Maintain Pluripotency and Proliferative Potential for Prolonged Periods of Culture," Dev Biol, 227:271-278 (2000). As stated in the

Materials and Methods section on page 272, at the top of the second column:

> To confirm growth from single cells for the derivation of clonal ES cell lines, individual cells were selected by direct observation under a stereomicroscope and transferred by micropipette to individual wells of a 96-well plate containing mouse embryonic fibroblast feeders with medium containing 20% serum replacer and 4 ng/ml bFGF.

Under these very diluted conditions (1 cell/well), the cloning efficiency was extremely poor. As stated in the last paragraph on page 272, even using a serum replacer, or change in the addition of bFGF, did not result in an efficiency above 1% (see Table 1 at the top of page 273).

The authors indicate in their discussion in the first full paragraph on page 277:

> Because current culture conditions are suboptimal, a significant percentage of the ES cells die at each passage, even when they are passaged in clumps.

This suggests that individually passaged cells are even more vulnerable.

From the above, it can be learned that using very "diluted" conditions of 1 cell/well, for example, results in extremely low (less than 1%) survival. In order to reach stable transfection, which requires prolonged cloning periods, less "diluted" conditions are needed as only these will enable satisfactory cloning efficiency. It could not have

necessarily been predicted with a reasonable predictability of success that one would be able to get a substantially pure, stably transformed cell line if the transfection yield was too small. Stable transfection can only be achieved using transfection protocols giving a good yield. If the yield is too small, then once you put the transfected cells onto culture plates with neomycin in order to kill off the non-stably transfected cells, if one is only left with an extremely small number of colonies, it could not be predicted to survive because of this dilution phenomenon. Therefore, it is critical to have a sufficient yield of stably transformed cells to be able to expect that, among those that are transiently transformed, one will have a sufficient number of stably transformed cells that they will sufficiently populate the culture disc with neomycin that they will grow and avoid this dilution problem. Zwaka and Thompson (2003), of record in this case, establish that, with electroporation of human cells, one gets only a 10^{-7} efficiency. One of ordinary skill in the art would not expect this small number to necessarily produce substantially pure, stably transformed cell lines, particularly in view of the dilution issues.

It should be noted that Smith has absolutely no examples with human embryonic stem cells. Indeed, all of the examples use murine cells, which are much easier to deal with

than human stem cells. While Smith lists a number of means of introducing selectable markers into cells, at column 2, lines 61-64, the only method used in the examples is electroporation. In order to anticipate, a reference must show every feature of the claim. The examiner is apparently taking the position that one following the procedure of Smith will inherently obtain substantially pure, stably transformed human embryonic cell lines. However, in order for an anticipation rejection to be based on inherency, such inherency must be certain. See MPEP §2112.IV, which states:

> The fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993) (reversed rejection because inherency was based on what would result due to optimization of conditions, not what was necessarily present in the prior art); *In re Oelrich*, 666 F.2d 578, 581-82, 212 USPQ 323, 326 (CCPA 1981). "To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.'" [emphasis original]

Here, the fact that a substantially pure, stably transfected population of pluripotent human embryonic stem cells could possibly occur if human cells are selected from

the varying kinds of animal cells disclosed in Smith and if a proper selection of introduction means is used from the myriad of such means described in Smith, does not establish that the claimed subject matter of claim 36 is inherently disclosed in Smith. Inherency, for the purposes of anticipation, is not established by probabilities or possibilities. In relying upon the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristics necessarily flow from the teachings of the applied prior art, *Ex parte Levy*, 17 USPQ2d 1461, 1464 (Bd Pat & Inter 1990). The mouse tests using electroporation certainly do not establish that obtaining a substantially pure, stably transfected population of pluripotent human embryonic stem cells necessarily occurs following the procedures of Smith, particularly in view of scientific explanations provided above and evidenced by the Amit publication. Reconsideration and withdrawal of this anticipation rejection are therefore respectfully urged.

Claims 1-4, 6, 7, 11-16, 36 and 59 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Smith in view of Fasbender for the reasons of record. The examiner recognizes that Smith does not teach the formulation of the polynucleotide with a cation non-lipid polymer

transfection reagent for introduction into the stem cells. The examiner states that Fasbender teaches methods of transfecting various cell types utilizing complexes of cationic molecules and adenovirus, which was found to enhance gene transfer *in vitro*. The examiner states that Fasbender teaches that the expression of reporter genes was increased in the cultured cells when they were transfected with the combination of viral vector and cationic molecules. Thus, the examiner considers the claimed invention as a whole to be clearly *prima facie* obvious at the time the claimed invention was made. In response to applicants' arguments and the previously submitted declarations of Prof. Benvenisty, the examiner states that applicants are arguing limitations that are not within the scope of the invention as there is no yield requirement, percentage of cells or efficiency that must be acquired to practice the invention. The examiner agreed with applicants' arguments that there would be no reason to use transfection reagents with electroporation, but with regard to applicants' arguments regarding using adenoviral vectors, the examiner states that applicants are arguing limitations that are not in the claims. This rejection is respectfully traversed.

In order to avoid this rejection, the claims have now been amended in order to insert the parameters that the

examiner states were missing. Claims 1 and 11 have now been amended to specify that the method of the present invention is one for altering gene expression in a population of human embryonic stem cells, with a transfection efficiency greater than that obtainable by means of electroporation. The introduction step specifies that the transfection efficiency must be greater than that obtainable by electroporation. This requirement is supported, for example, by the present specification at page 11, lines 30-33, where it states:

> In contrast to mouse embryonic stem cells, we found that although human ES cells can be transfected by electroporation, improved results were obtained by transfection in the presence of cationic polymers.

This is further supported by Figure 1 and the explanation thereof in the present specification, and the further explanation provided in the second Benvenisty Declaration of record in this case. Accordingly, the present claims now require a yield greater than that obtainable by electroporation. There is certainly nothing in Smith or Fasbender that would suggest that by using the transfection reagents required by the present claims, one would be able to get a transfection efficiency greater than that of electroporation when using human embryonic stem cells.

Furthermore, claims 1 and 11 have been amended to specify that the nucleic acid introduced into the human

embryonic stem cells does not contain viral genes. This limitation is supported, for example, at page 4, line 24, of the present specification, which states:

> In embodiments of the method, the nucleic acid may not contain viral genes.

Accordingly, the present claims now exclude the possible use of adenovirus as an introduction agent, such as is used in Fasbender.

Thus, no combination of Smith with Fasbender could make obvious the use of the claimed transfection reagents without the introduction of viral genes into the cell and in a manner such that the transfection efficiency is greater than that obtainable by electroporation. Reconsideration and withdrawal of this rejection are therefore respectfully urged.

The examiner states that the Benvenisty Declaration under 37 C.F.R. §1.132 filed April 20, 2005, was not considered proper because it was not signed. The examiner has requested applicants to file the signed declaration in response to this Office action.

Applicants' records show that a signed copy of the declaration was mailed on May 6, 2005, and received by the PTO on May 9, 2005. Nonetheless, another copy of the signed declaration is attached hereto. As all of the attachments to the declaration have previously been submitted, an additional copy of such attachments is not being provided at this time so

as not to clutter the record. Clearly the attachments referred to in the attached signed copy of the declaration are those that were attached to the unsigned copy of the declaration that has been previously filed.

It is submitted that all of the claims now present in the case clearly define over the references of record. Reconsideration and allowance are therefore earnestly solicited.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant(s)

By _____
Roger L. Browdy
Registration No. 25,618

RLB:rd
Telephone No.: (202) 628-5197
Facsimile No.: (202) 737-3528
G:\BN\L\LUZZ\Benvenisty5\Pto\AmendmentE.doc

Developmental Biology 227, 271–278 (2000)
doi:10.1006/dbio.2000.9912, available online at http://www.idealibrary.com on IDEAL®

Clonally Derived Human Embryonic Stem Cell Lines Maintain Pluripotency and Proliferative Potential for Prolonged Periods of Culture

Michal Amit,* Melissa K. Carpenter,† Margaret S. Inokuma,† Choy-Pik Chiu,† Charles P. Harris,‡ Michelle A. Waknitz,§ Joseph Itskovitz-Eldor,* and James A. Thomson§,¶

**Department of Obstetrics and Gynecology, The Rambam Medical Center, and Faculty of Medicine, Technion, Haifa 31096, Israel; †Geron Corporation, 230 Constitution Drive, Menlo Park, California 94025; ‡Texas Children's Hospital, Baylor College of Medicine, Houston, Texas 77030; and §The Wisconsin Regional Primate Research Center and ¶Department of Anatomy, School of Medicine, University of Wisconsin, Madision, Wisconsin 53715*

Embryonic stem (ES) cell lines derived from human blastocysts have the developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture. Here we describe the clonal derivation of two human ES cell lines, H9.1 and H9.2. At the time of the clonal derivation of the H9.1 and H9.2 ES cell lines, the parental ES cell line, H9, had already been continuously cultured for 6 months. After an additional 8 months of culture, H9.1 and H9.2 ES cell lines continued to: (1) actively proliferate, (2) express high levels of telomerase, and (3) retain normal karyotypes. Telomere lengths, while somewhat variable, were maintained between 8 and 12 kb in high-passage H9.1 and H9.2 cells. High-passage H9.1 and H9.2 cells both formed teratomas in SCID-beige mice that included differentiated derivatives of all three embryonic germ layers. These results demonstrate the pluripotency of single human ES cells, the maintenance of pluripotency during an extended period of culture, and the long-term self-renewing properties of cultured human ES cells. The remarkable developmental potential, proliferative capacity, and karyotypic stability of human ES cells distinguish them from adult cells. © 2000 Academic Press

Key Words: human embryonic stem cells; basic fibroblast growth factor; cloning; telomeres.

INTRODUCTION

Human pluripotent cell lines have been derived from preimplantation embryos (embryonic stem cell lines, ES cells; Reubinoff *et al.*, 2000; Thomson *et al.*, 1998) and from fetal germ cells (embryonic germ cell lines, EG cells; Shamblott *et al.*, 1998) that for prolonged periods of culture maintain a stable developmental potential to form advanced derivatives of all three embryonic germ layers. Human ES cell lines have widespread implications for human developmental biology, drug discovery, drug testing, and transplantation medicine. For example, current knowledge of the postimplantation human embryo is largely based on a limited number of static histological sections, and because of ethical considerations, the underlying mechanisms that control the developmental decisions of the early human embryo remain essentially unexplored. Although the mouse is the mainstay of experimental mammalian developmental biology, there are significant differences between early mouse and human development. These differences are especially prominent in the extraembryonic membranes, in the placenta, and in the arrangement of the germ layers at the time of gastrulation. The yolk sac, for example, is a robust, well-vascularized extraembryonic tissue that is important throughout mouse gestation, but in the human embryo, the yolk sac is essentially a vestigial structure during later gestation (Kaufman, 1992; O'Rahilly and Muller, 1987). Human ES cells should provide important new insights into the differentiation and function of tissues that differ significantly between mice and humans.

In addition to advancing basic developmental biology, human ES cells should have practical, applied uses. The differentiated derivatives of human ES cells could be used for: (1) identification of gene targets for new drugs, (2)

0012-1606/00 $35.00

testing the toxicity or teratogenicity of new compounds, and (3) transplantation to replace cell populations destroyed by disease. Potential conditions that might be treated by the transplantation of ES cell-derived cells include Parkinson's disease, cardiac infarcts, juvenile-onset diabetes mellitus, and leukemia (Gearhart, 1998; Rossant and Nagy, 1999). However, the scientific and therapeutic potential of human ES cells critically depends on their long-term proliferative capacity, their developmental potential after prolonged culture, and their karyotypic stability. The originally described human ES and EG cell lines were not clonally derived from single cells and thus pluripotency could be demonstrated only for a population of cells (Reubinoff *et al.*, 2000; Shamblott *et al.*, 1998; Thomson *et al.*, 1998). Therefore, the formal possibility existed that within the population of homogeneous-appearing cells there were actually multiple precursor or stem cells committed to different lineages and that no single cell was capable of forming derivatives of all three embryonic germ layers. The human ES cells we previously derived were isolated and propagated as small clumps because nonhuman primate ES cell colonies dissociated to single cells plate at a very low efficiency (Thomson *et al.*, 1995; Thomson and Marshall, 1998).

Here we describe conditions for clonally deriving human ES cell lines. Two clonally derived human ES cell lines proliferated for a period of at least 8 months after clonal derivation (population doubling (PD) 286 from initial derivation of the parental cell line) and maintained the ability to differentiate to advanced derivatives of all three embryonic germ layers. Both clonal human ES cell lines expressed high levels of telomerase and maintained terminal restriction fragment (TRF) lengths between 8 and 12 kb. In contrast, normal human somatic cells gradually lose telomeric DNA and senesce after 50–80 PD when TRF lengths are about 5–7 kb. Thus, these results clearly demonstrate the pluripotency of single human ES cells and demonstrate the remarkable proliferative capacity of these cells.

MATERIALS AND METHODS

The derivation, routine culture, and characterization of the human ES cell line H9 were previously described (Thomson *et al.*, 1998). Human ES cells were plated on irradiated (35 gray γ irradiation) mouse embryonic fibroblasts. Culture medium for the present work consisted of 80% KnockOut Dulbecco's modified Eagle's medium, an optimized medium for mouse ES cells (Gibco BRL, Rockville, MD), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 1% nonessential amino acids stock (Gibco BRL), supplemented with either 20% fetal bovine serum (HyClone, Logan, UT) or 20% KnockOut SR, a serum replacer optimized for mouse ES cells (Gibco BRL). The components of KnockOut SR have been published elsewhere (Price *et al.*, 1998). In initial cloning experiments, medium was supplemented with either serum or serum replacer and was used either with or without human recombinant basic fibroblast growth factor (bFGF; 4 ng/ml). For prolonged culture, the serum-free medium required supplementation with bFGF.

To determine cloning efficiency, H9 cells were dissociated to single cells for 7 min with 0.05% trypsin/0.25% EDTA, washed by centrifugation, and plated on mitotically inactivated mouse embryonic fibroblasts (10^5 ES cells in triplicate wells of 6-well plates). To confirm growth from single cells for the derivation of clonal ES cell lines, individual cells were selected by direct observation under a stereomicroscope and transferred by micropipette to individual wells of a 96-well plate containing mouse embryonic fibroblast feeders with medium containing 20% serum replacer and 4 ng/ml bFGF. Clones were expanded by routine passage every 7 days with 1 mg/ml collagenase type IV (Gibco BRL).

For karyotype analysis, either standard G banding or multicolor spectral karyotyping (SKY) was performed (Schrock *et al.*, 1996). For SKY analysis, the SKY H-10 kit was used according to the manufacturer's instructions (Applied Spectral Imaging, Inc., Carlsbad, CA). Metaphase figures were dropped onto clean glass slides and treated with combinatorially labeled whole genome painting probes. After stringent washes in 50% formamide, images of metaphase spreads were captured using the Applied Spectral Imaging spectrophotometer and SKY software on a Zeiss Axioplan II microscope. Karyotypes were analyzed and arranged with combined software processing of the image and reverse DAPI banding. For each SKY sample, 5 metaphases were captured and analyzed completely, and 20 metaphases were captured for modal number determination.

Prior to measurement of telomerase activity and telomere length, ES cells expressing TRA-1-60 (a marker of undifferentiated human ES cells) were selected from cultures grown on irradiated mouse embryonic fibroblasts. Cells were dissociated using 0.2% EDTA and then incubated with a monoclonal antibody against TRA-1-60 (gift from Peter Andrews). After washing, cells were incubated with goat anti-mouse IgM-conjugated magnetic microbeads and processed through a MACS magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) (Walz *et al.*, 1995). In the samples used for these experiments, greater than 90% of the population was positive for TRA-1-60 using flow cytometric analysis. Telomerase activity was measured using the telomeric repeat amplification protocol (TRAP) assay as described (Kim *et al.*, 1994; Weinrich *et al.*, 1997). TRF size was determined using Southern hybridization essentially as described (Allsopp *et al.*, 1992; Harley *et al.*, 1990; Vaziri *et al.*, 1993).

For teratoma formation, H9.1 and H9.2 cells, cultured for 6 months after cloning, were injected into the rear leg muscle of 4-week-old male SCID-beige mice (eight mice total). Cell numbers ranged from 2.5×10^6 cells to 7.5×10^6 cells per injection. Three to four months after injection the mice were sacrificed and the resulting teratomas examined histologically.

RESULTS

To demonstrate the long-term pluripotency and replicative immortality of single human ES cells, we have derived clonal human ES cell lines. These clonal lines have been maintained for over 1 year *in vitro*, and proliferation rate, karyotypes, teratoma formation, telomere length, and telomerase activity have been examined.

The cloning efficiency of human ES cells was extremely poor under previously described culture conditions that included serum. A several-fold increase in cloning efficiency of human ES cells was consistently observed when serum-free medium was used instead of serum-containing

TABLE 1
Cloning Efficiency of H9 Human ES Cells[a]

	(−) bFGF	(+) bFGF (4 ng/ml)
20% Serum	240 ± 28 (0.24)*	260 ± 12 (0.26)*
20% Serum replacer	633 ± 43 (0.63)†	826 ± 61 (0.83)‡

[a] Values are expressed as the mean numbers of colonies resulting from 10^5 individualized ES cells plated ±SE (figures in parentheses represent percentage colony cloning efficiency).

*,†,‡ Means with different superscripts differ significantly ($P < 0.05$, Tukey–Kramer HSD.

medium (Table 1). The addition of bFGF to the medium altered the morphology of human ES cells, resulting in smaller cells in tighter colonies (Fig. 1A). The long-term culture of human ES cells in the presence of serum does not require the addition of exogenous bFGF and the addition of bFGF to serum-containing medium did not significantly increase human ES cell cloning efficiency (Table 1). However, in serum-free medium, bFGF increased the initial cloning efficiency of human ES cells and bFGF was required for continued undifferentiated proliferation. In serum-free medium lacking bFGF, human ES cells became uniformly differentiated by 2 weeks after plating (Fig. 1B).

To avoid the possibility that some of the colonies that grew were not from individual cells, but were from rare, small clumps of cells remaining after dissociation, the H9 cell line was recloned by placing cells individually into wells of a 96-well plate under direct microscopic observation. Of 384 H9 cells (at PD 122) plated into 96-well plates, 2 clones were successfully expanded (H9.1 and H9.2). Both of these clones were subsequently cultured continuously in medium supplemented with serum replacer and bFGF. H9.1 and H9.2 cells maintained a normal XX karyotype even after more than 8 months of continuous culture after clonal derivation (Fig. 2).

Population doubling time was measured in the parent line and in both clonal lines and no significant differences were found between the parent line and the clonal lines. The average population doubling time for 10 separate determinations was 35.3 ± 2.0 h (mean ± standard error of the mean). Because of the considerable cell death observed in these human ES cell cultures, this population doubling time may underestimate the replication rate of the ES cells that survive. In addition, evaluation of the karyotype in the H9 parental population 6 months after derivation (PD 122) presented a normal XX karyotype by standard G-banding techniques (20 chromosomal spreads analyzed). However, 7 months after derivation, in a single karyotype preparation, 4/20 spreads demonstrated random abnormalities; one with a translocation to chromosome 13 short arm, one with an inverted chromosome 20, one with a translocation to the number 4 short arm, and one with multiple fragmentation. Subsequently, at 8, 10, and 12.75 months after derivation (PD 260), H9 cells exhibited normal karyotypes in all 20 chromosomal spreads examined. Both H9.1 and H9.2 also exhibited normal karyotypes 8 months after derivation.

Telomerase activity was high in H9, H9.1, and H9.2 cells at all time points examined (Fig. 3), ranging from about 52 to 196% of that found in H1299, a lung tumor cell line. In these experiments, the human ES cells were separated from fibroblast feeders by magnetic bead sorting to greater than 90% of the cells as determined by Tra-1-60 expression. Even though telomerase activity was also found in the mouse feeder cells (MEF, Fig. 3), the activity in MEF is 4–15% of that found in human ES cells. Therefore, it is unlikely that

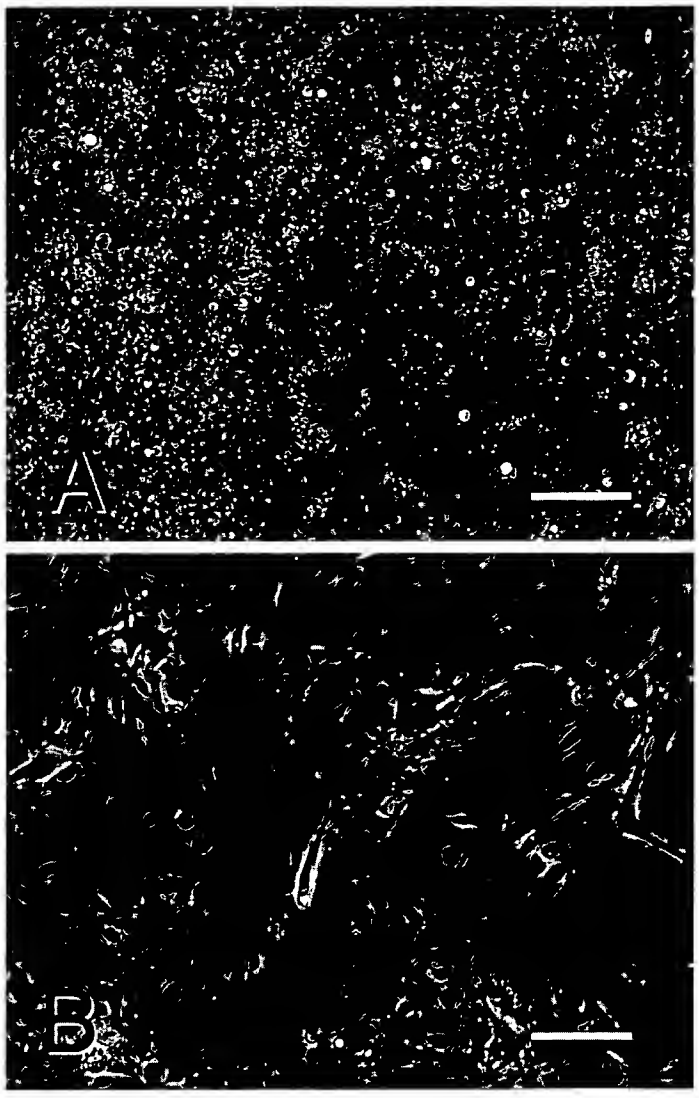

FIG. 1. H9 human ES cells cultured for 14 days in serum-free medium in the presence (4 ng/ml) (A) or absence (B) of bFGF. ES cells plated in serum-free medium in the presence of bFGF continued active, undifferentiated proliferation throughout the culture period. ES cells plated in the absence of bFGF uniformly differentiated into a flattened, epithelial morphology by the end of the 14-day culture period. Bar, 200 μm.

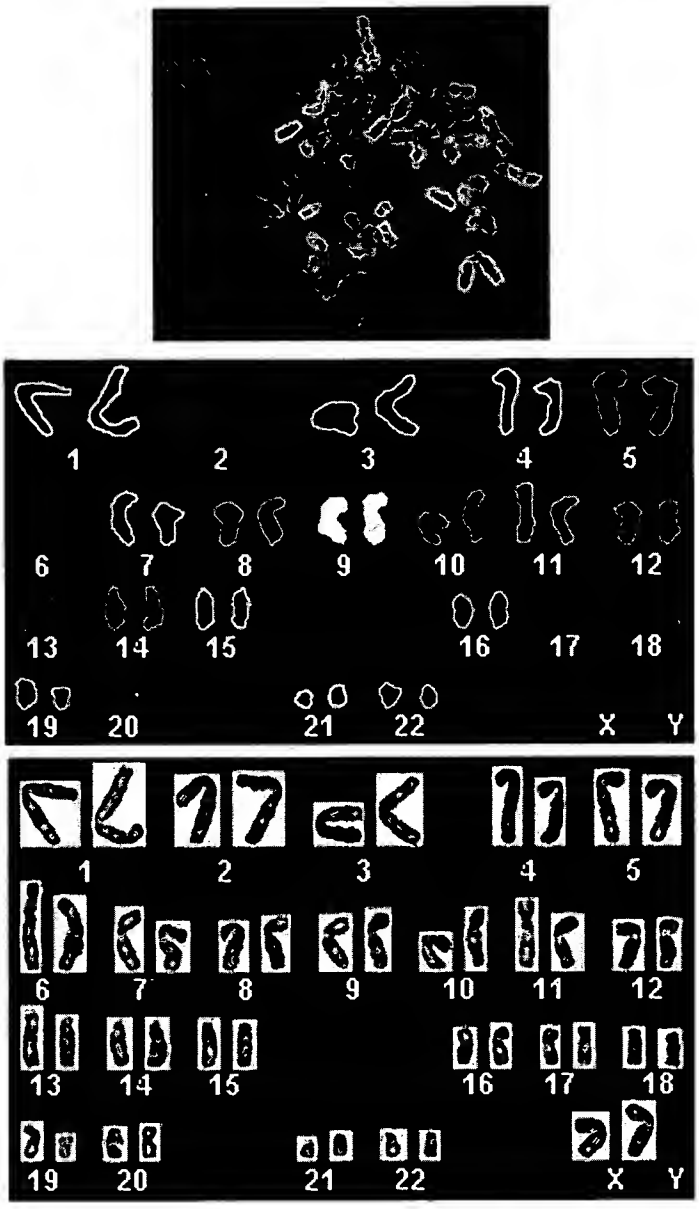

FIG. 2. Metaphase preparation from H9.2 at 8 months (PD 175) of continuous culture. Captured metaphase spectral image (top); classified processed image (middle); reverse DAPI image (bottom). Karyotype: 46, XX normal female.

the telomerase activity seen in the human ES cells is entirely due to the <10% contamination from the feeders.

In general, the presence of telomerase activity is associated with a maintenance of telomere length. H9.1 and H9.2 clones showed roughly stabilized telomere lengths, between 8 and 12 kb. As seen in other populations of telomerase-positive cells, changes in telomere length did not correlate with telomerase activity. At PD 57 after clonal derivation, H9.2 cells had a telomere length of about 8 kb which increased to 13 kb by PD 143. H9.1 cells showed an initial decrease in telomere length followed by an increase to about 11 kb at PD 123. On the other hand, the parental cell line, H9, demonstrated an initial decrease in telomere length from 14 kb at PD 71 (passage 15) to 9 kb at PD 200 (passage 42) (Fig. 3). Cells at later PD will need to be examined to determine if telomeres continue to shorten. However, to date, H9 is still proliferating well at PD 304.

The H9.1 and H9.2 clones maintained the potential to form derivatives of all three embryonic germ layers after long-term culture under serum-free conditions. After 6 months of culture (PD 122), H9.1 and H9.2 clones were confirmed to have normal karyotypes and were then injected into SCID-beige mice. Both H9.1 and H9.2 cells formed teratomas that contained derivatives of all three embryonic germ layers, including gut epithelium (endoderm); embryonic kidney, striated muscle, smooth muscle, bone, and cartilage (mesoderm); and neural tissue (ectoderm) (Fig. 4). The range of differentiation observed within the teratomas of the high-passage H9.1 and H9.2 cells was comparable to that observed in teratomas formed by low-passage parental H9 cells.

DISCUSSION

This report addresses two critical issues: the pluripotentiality and the long-term replicative capacity of clonal human ES cell lines. The previously described human ES cell lines appeared to be a morphologically homogeneous population of cells and expressed cell surface markers characteristic of clonally derived primate ES cells (Reubinoff *et al.*, 2000; Thomson *et al.*, 1998, 1995; Thomson and Marshall, 1998). However, because these human ES cell lines were not clonally derived, pluripotency could be demonstrated only for a population of cells and not for individual ES cells. Our successful cloning of human ES cell lines demonstrates that individual cells are indeed pluripotent and that such individual pluripotent ES cells were present after at least 6 months of continuous culture of the parental H9 cell line. Furthermore, these clonally derived cell lines demonstrated a developmental potential after more than 6 months of additional culture (12 months after the derivation of the parental cell line) that was comparable to low-passage ES cells.

The telomere hypothesis suggests that critical telomere shortening signals cell senescence (Harley *et al.*, 1990; Vaziri *et al.*, 1993) and that telomerase can prevent senescence by maintenance of telomere length (Bodnar *et al.*, 1998; Jiang *et al.*, 1999). This hypothesis is supported by the presence of high telomerase activity in cells with extensive replicative capacity such as germ cells or tumor cells (Chiu and Harley, 1997). The telomerase activity seen in human ES cells, especially in the cell lines which have survived approximately 300 population doublings, i.e., four to six times the life span of normal somatic cells, suggests that these cells are immortal.

Telomere lengths in cloned human ES cells showed some variations from passage to passage, but are stabilized over-

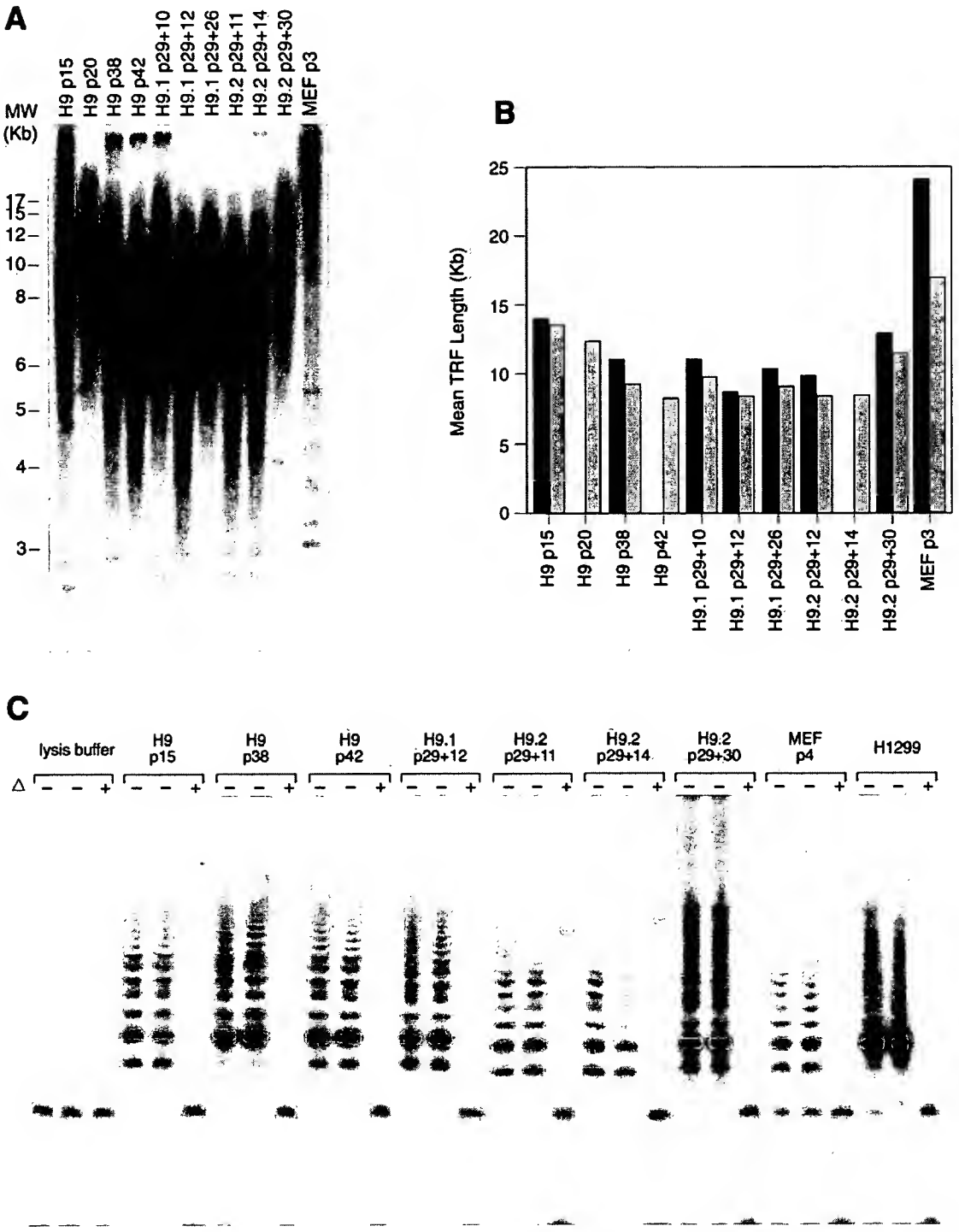

FIG. 3. Telomere length and telomerase activity in human ES cells. (A) Southern analysis of terminal restriction fragment (TRF) from the H9 parent line and two clonal lines, H9.1 and H9.2, at different times *in vitro*. Passage number for the clones is represented as number of passages following subcloning at passage 29 (p29 + *X*). Cells were passaged at 7-day intervals. MEF indicates irradiated mouse primary embryonic fibroblasts. (B) Mean TRF lengths. Telomere length was quantified using a PhosphorImager and ImageQuant software. The mean TRF length for each lane is an integral function based on the densitometric readings in reference to the standards for each gel (A). Mean TRF length was calculated from replicate analyses: light gray bars represent data for gel A, dark gray bars represent replicate analysis of selected samples from gel A. (C) TRAP analysis of telomerase activity in human ES cells. The TRAP assay is a primer extension assay in which telomerase synthesizes telomeric repeats onto oligonucleotide primers. The telomerase extension products serve as a template for PCR amplification. The laddering in the TRAP gel represents increasing numbers of telomeric repeats in which radionucleotides are incorporated. Samples were run in triplicate with the third sample as a negative control in which cell lysates were heat inactivated prior to the assay (indicated by the Δ). The positive control was a cell extract from the telomerase-expressing tumor line H1299 cells.

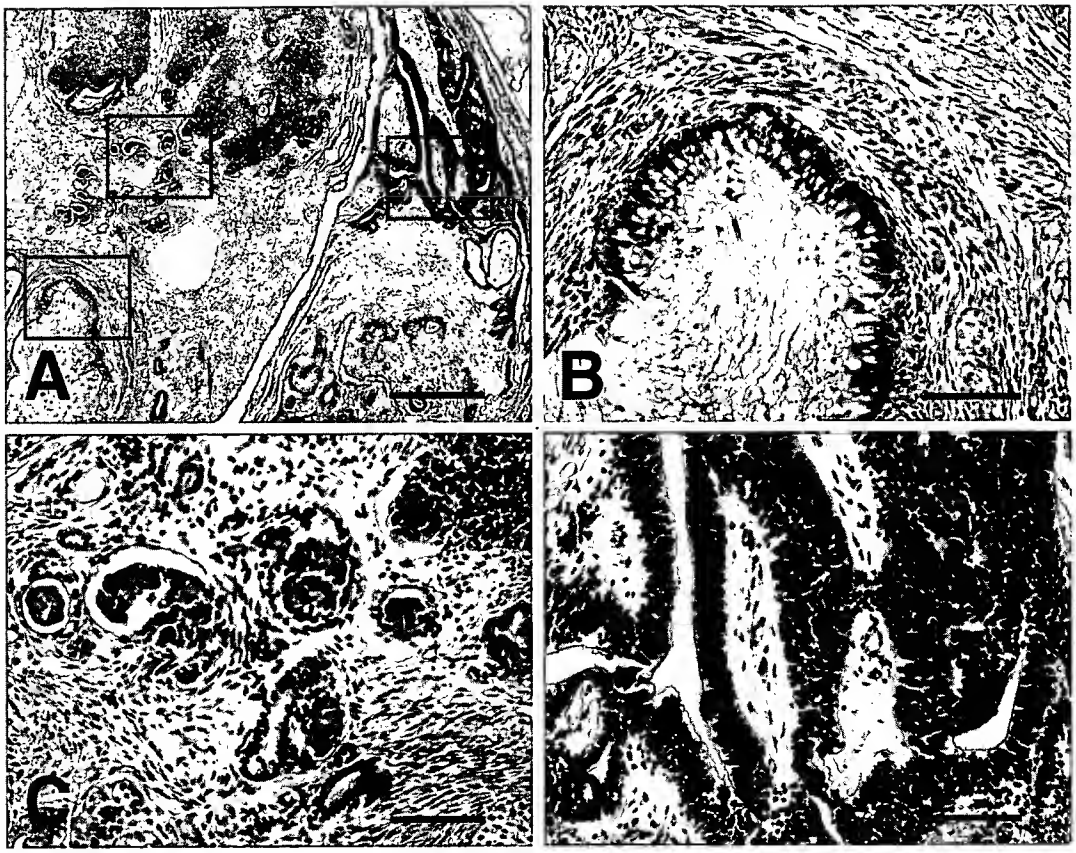

FIG. 4. Teratomas from H9.1 cells. Approximately 5×10^6 H9.1 cells that had been cultured for 6 months (PD 131) after clonal derivation were injected into the hind leg musculature of a SCID-beige mouse. The resulting tumor was harvested and examined 3 months after injection. (A) Low-power view of a field that exhibits differentiated derivatives of all three embryonic germ layers. Areas enclosed in boxes are enlarged in B, C and D (bar, 500 μm). (B) Gut epithelium with adjacent smooth muscle (bar, 100 μm). (C) Embryonic glomeruli and renal tubules (bar, 100 μm). (D) Neural epithelium (bar, 100 μm).

all. This could be due to heterogeneity in the differentiated state of the cultures or could be due to variation or drift in the abundance of factors which establish the equilibrium point for telomere length, in which gain due to telomerase activity matches loss due to incomplete replication and degradation (Chiu *et al.*, 1996; Vaziri *et al.*, 1994). The parental H9 ES cell line showed a decrease in telomere length with increasing population doublings. Despite the telomere shortening, H9 cells are still proliferating well at PD 304. Other immortalized cells also demonstrate telomere shortening in the presence of telomerase and continued proliferation. For example, endothelial cells engineered to express telomerase continue to proliferate but show decreased telomere length which eventually stabilizes (Yang *et al.*, 1999). We are continuing to monitor telomere length in H9 cells.

Serum is a complex mixture that can contain compounds both beneficial and detrimental to human ES cell culture. Different serum batches vary widely in their ability to support vigorous undifferentiated proliferation of human ES cells. Replacing serum with defined components should reduce the variability of experiments associated with serum batch variation and should allow more carefully defined differentiation studies. The consistently lower cloning efficiency in medium containing serum suggests the presence of compounds that are detrimental to stem cell survival when the cells are dispersed to single cells. Although we were able to sustain human ES cells for prolonged periods in a commercially available serum substitute, substantial improvements to the serum-free culture of human ES cells are still needed. Most important, the present culture conditions support a cloning efficiency of human ES cells (<1%) that is considerably lower than the cloning efficiency of mouse ES cells. Unless cloning efficiency is increased significantly, techniques standard to mouse ES cells, such as homologous recombination, will be very difficult to apply to human ES cells.

The fibroblast feeder layer remains the most poorly defined component of the human ES cell culture environment. Unlike undifferentiated mouse ES cells, which can be cultured using leukemia inhibitory factor (LIF) in the absence of fibroblasts (Smith *et al.*, 1988; Williams *et al.*,

1988), under current culture conditions, addition of LIF to the medium does not allow the culture of human ES cells in the absence of feeder layers (Thomson *et al.*, 1998). If LIF and bFGF are added in combination, human ES cells are still lost to differentiation in the absence of fibroblasts (not shown). To date we have also been unable to demonstrate beneficial effects of exogenously added LIF in the presence of fibroblasts (not shown). However, fibroblasts can produce LIF, and given the importance of LIF in the culture of human EG cells (Shamblott *et al.*, 1998), further examination of the role of LIF in human ES cell self-renewal is warranted. Identifying the factors that the fibroblasts produce that promote human ES cell renewal will be critical to the large-scale growth of ES cells, because the feeder layers are labor intensive to prepare and because variation between batches of fibroblasts can introduce undesirable variation and complexity to experiments. It is unknown, for example, whether the beneficial effects of bFGF on undifferentiated human ES cell growth are mediated through the fibroblasts, the ES cells, or both.

Because current culture conditions are suboptimal, a significant percentage of the ES cells die at each passage, even when they are passaged in clumps. For this reason, there is selective pressure for any rare cell population that has an enhanced survival at splitting or an increased rate of proliferation. Improved passaging and culture conditions that increase cloning efficiency should reduce this selective pressure. Given this selective pressure, the karyotypic stability of human ES cells is remarkable, but clones with abnormal karyotypes and a selective growth advantage should eventually take over a culture. Therefore, it is likely that it will be necessary to periodically subclone human ES cells to maintain a euploid population. In a single sample of H9 cells at 7 months of culture, we observed a subpopulation (4 of 20 chromosomal spreads) with abnormal karyotypes. These were the only abnormal karyotypes we observed, and curiously, subsequent karyotypes of the uncloned H9 cells at 8, 10, and 12.75 months of culture revealed 20 of 20 normal chromosomal spreads. We have yet to observe abnormal karyotypes in the H9.1 or H9.2 cells, even at 8 months after their clonal derivation. Thus, although the useful long-term expansion of human ES cells could be somewhat limited by a need to subclone periodically, our results suggest that this need will be infrequent. Ultimately, the slow accumulation of genetic mutations may place an upper limit on the useful long-term culture of human ES cells. Although these considerations may place some practical constraints on the maintenance of human ES cells, the potential uses of ES cells as biological research tools and as therapeutic agents remain unparalleled.

ACKNOWLEDGMENTS

We thank Peter Andrews for the gift of the TRA-1-60 antibody; Yoko Oei and Kathy Ng for technical assistance; and Jane Lebkowski, Calvin Harley, and Ruth Singer for critically reading the manuscript. This work was supported by the Geron Corporation (Grant 133-BU18) and the fund for Medical Research and Development of Infrastructure and Health Services, Rambam Medical Center.

REFERENCES

Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W., and Harley, C. B. (1992). Telomere length predicts replicative capacity of human fibroblasts. *Proc. Natl. Acad. Sci. USA* 89, 10114–10118.

Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S., and Wright, W. E. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science* 279, 349–352.

Chiu, C. P., Dragowska, W., Kim, N. W., Vaziri, H., Yui, J., Thomas, T. E., Harley, C. B., and Lansdorp, P. M. (1996). Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow. *Stem Cells* 14, 239–248.

Chiu, C. P., and Harley, C. B. (1997). Replicative senescence and cell immortality: The role of telomeres and telomerase. *Proc. Soc. Exp. Biol. Med.* 214, 99–106.

Gearhart, J. (1998). New potential for human embryonic stem cells. *Science* 282, 1061–1062.

Harley, C. B., Futcher, A. B., and Greider, C. W. (1990). Telomeres shorten during ageing of human fibroblasts. *Nature* 345, 458–460.

Jiang, X. R., Jimenez, G., Chang, E., Frolkis, M., Kusler, B., Sage, M., Beeche, M., Bodnar, A. G., Wahl, G. M., Tlsty, T. D., and Chiu, C. P. (1999). Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. *Nat. Genet.* 21, 111–114.

Kaufman, M. H. (1992). "The Atlas of Mouse Development." Academic Press, London.

Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science* 266, 2011–2015.

O'Rahilly, R., and Muller, F. (1987). "Developmental Stages in Human Embryos." Carnegie Inst. of Washington, Washington, DC.

Price, P. J., Goldsborough, M. D., and Tilkins, M. L. (1998). Embryonic stem cell serum replacement. International Patent Application WO98/30679.

Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A., and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: Somatic differentiation in vitro. *Nat. Biotechnol.* 18, 399–404.

Rossant, J., and Nagy, A. (1999). In search of the tabula rasa of human cells. *Nat. Biotechnol.* 17, 23–24.

Schrock, E., du Manoir, S., Veldman, T., Schoell, B., Wienberg, J., Ferguson-Smith, M. A., Ning, Y., Ledbetter, D. H., Bar-Am, I., Soenksen, D., Garini, Y., and Ried, T. (1996). Multicolor spectral karyotyping of human chromosomes. *Science* 273, 494–497.

Shamblott, M. J., Axelman, J., Wang, S., Bugg, E. M., Littlefield, J. W., Donovan, P. J., Blumenthal, P. D., Huggins, G. R., and Gearhart, J. D. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc. Natl. Acad. Sci. USA* 95, 13726–13731.

Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M., and Rogers, D. (1988). Inhibition of pluripotential

embryonic stem cell differentiation by purified polypeptides. *Nature* **336**, 688–690.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147.

Thomson, J. A., Kalishman, J., Golos, T. G., Durning, M., Harris, C. P., Becker, R. A., and Hearn, J. P. (1995). Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. USA* **92**, 7844–7848.

Thomson, J. A., and Marshall, V. S. (1998). Primate embryonic stem cells. *Curr. Top. Dev. Biol.* **38**, 133–165.

Vaziri, H., Dragowska, W., Allsopp, R. C., Thomas, T. E., Harley, C. B., and Lansdorp, P. M. (1994). Evidence for a mitotic clock in human hematopoietic stem cells: Loss of telomeric DNA with age. *Proc. Natl. Acad. Sci. USA* **91**, 9857–9860.

Vaziri, H., Schachter, F., Uchida, I., Wei, L., Zhu, X., Effros, R., Cohen, D., and Harley, C. B. (1993). Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. *Am. J. Hum. Genet.* **52**, 661–667.

Walz, T. M., Malm, C., Nishikawa, B. K., and Wasteson, A. (1995). Transforming growth factor-alpha (TGF-alpha) in human bone marrow: Demonstration of TGF-alpha in erythroblasts and eosinophilic precursor cells and of epidermal growth factor receptors in blastlike cells of myelomonocytic origin. *Blood* **85**, 2385–2392.

Weinrich, S. L., Pruzan, R., Ma, L., Ouellette, M., Tesmer, V. M., Holt, S. E., Bodnar, A. G., Lichtsteiner, S., Kim, N. W., Trager, J. B., Taylor, R. D., Carlos, R., Andrews, W. H., Wright, W. E., Shay, J. W., Harley, C. B., and Morin, G. B. (1997). Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. *Nat. Genet.* **17**, 498–502.

Williams, R., Hilton, D., Pease, S., Wilson, T., Stewart, C., Gearing, D., Wagner, E., Metcalf, D., Nicola, N., and Gough, N. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* **336**, 684–687.

Yang, J., Chang, E., Cherry, A. M., Bangs, C. D., Oei, Y., Bodnar, A., Bronstein, A., Chiu, C. P., and Herron, G. S. (1999). Human endothelial cell life extension by telomerase expression. *J. Biol. Chem.* **274**, 26141–8.

Received for publication June 8, 2000
Accepted August 18, 2000
Published online October 13, 2000